

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09592 A1

(51) International Patent Classification⁷: **G01N 21/64,**
33/543

Dartmouth Place, LaCanada-Flintridge, CA 91011 (US).
LANSFORD, Russell, D.; 522 E. Miramar Avenue, Clare-
mont, CA 91711 (US).

(21) International Application Number: PCT/US00/20591

(22) International Filing Date: 28 July 2000 (28.07.2000)

(74) Agent: REED, Derrick, W.; Christie, Parker & Hale, LLP,
P.O. Box 7068, Pasadena, CA 91109-7068 (US).

(25) Filing Language: English

(26) Publication Language: English

(84) Designated States (*regional*): European patent (AT, BE,
CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,
NL, PT, SE).

(30) Priority Data:
60/146,490 30 July 1999 (30.07.1999) US
60/164,504 9 November 1999 (09.11.1999) US

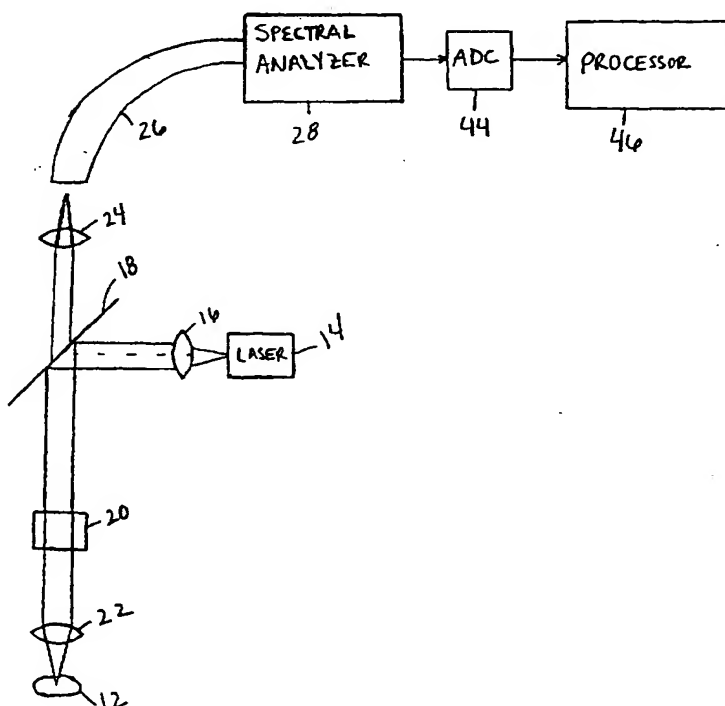
Published:
— With international search report.
— Before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments.

(71) Applicant: CALIFORNIA INSTITUTE OF TECH-
NOLOGY [US/US]; 1200 East California Boulevard,
Mail Stop 201-85, Pasadena, CA 91125 (US).

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(72) Inventors: BEARMAN, Gregory, H.; 974 East Eliza-
beth, Pasadena, CA 91104 (US). FRASER, Scott, E.; 503

(54) Title: SYSTEM AND METHOD FOR MONITORING CELLULAR ACTIVITY



(57) Abstract: A system and method for monitoring cellular activity in a cellular specimen. According to one embodiment, a plurality of excitable markers are applied to the specimen. A multi-photon laser microscope is provided to excite a region of the specimen and cause fluorescence to be radiated from the region. The radiating fluorescence is processed by a spectral analyzer to separate the fluorescence into respective wavelength bands. The respective bands of fluorescence are then collected by an array of detectors, with each detector receiving a corresponding one of the wavelength bands.



WO 01/09592 A1

| |
|-------------------|
| FP04-0318-00WO-HP |
| 04.11.16 |
| SEARCH REPORT |

1 **SYSTEM AND METHOD FOR MONITORING CELLULAR ACTIVITY**

5 **CROSS-REFERENCE TO RELATED APPLICATIONS**

 This application is based on provisional patent application numbers 60/146,490 filed July 30, 1999, and 60/164,504 filed November 9, 1999.

10 **STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT**

 The invention described herein was made in the performance of work under a NASA contract, and is subject to the provisions of Public Law 96-517 (35 USC 202) in which the Contractor has elected to retain title.

15 **FIELD OF THE INVENTION**

 The present invention relates to the monitoring of cellular activity through the use of excitable markers. More particularly, the invention relates to a system and method for using a plurality of fluorescent probes to monitor cellular activity.

20 **BACKGROUND OF THE INVENTION**

 Presently, fluorescence microscopy is one of the most widely used microscopy techniques, as it enables the molecular composition of the structures being observed to be identified through the use of fluorescently-labeled probes of high chemical specificity, such as antibodies. However, its use is mainly confined to studies of fixed specimens because of the difficulties of introducing antibody complexes into living specimens. For proteins that can be
25 extracted and purified in reasonable abundance, these difficulties can be circumvented by directly conjugating a fluorophore to a protein and introducing this back into a cell. It is believed that the fluorescent analogue behaves like the native protein and can therefore serve to reveal the distribution and behavior of this protein in the cell.

30 An exciting, new development in the use of fluorescent probes for biological studies has been the development of the use of naturally fluorescent proteins as fluorescent probes, such as green fluorescent protein (GFP). The gene for this protein has been cloned and can be transfected into other organisms. This can provide a very powerful tool for localizing regions in which a particular gene is expressed in an organism, or in identifying the location of a particular protein.
35 The beauty of the GFP technique is that living, unstained samples can be observed. There are presently several variants of GFP which provide spectrally distinct emission colors.

 Conventionally, fluorescence microscopy only worked well with very thin specimens or

1 when a thick specimen was cut into sections, because structures above and below the plane of
focus gave rise to interference in the form of out-of-focus flare. However, this can be overcome
by optical sectioning techniques, such as multi-photon fluorescence microscopy.

5 Multi-photon fluorescence microscopy involves the illumination of a sample with a
wavelength around twice the wavelength of the absorption peak of the fluorophore being used.
For example, in the case of fluorescein which has an absorption peak around 500nm, 900 nm
excitation could be used. Essentially no excitation of the fluorophore will occur at this
wavelength. However, if a high peak-power, pulsed laser is used (so that the mean power levels
10 are moderate and do not damage the specimen), two-photon events will occur at the point of
focus. At this point the photon density is sufficiently high that two photons can be absorbed by
the fluorophore essentially simultaneously. This is equivalent to a single photon with an energy
equal to the sum of the two that are absorbed. In this way, fluorophore excitation will only occur
at the point of focus (where it is needed) thereby eliminating excitation of the out-of-focus
15 fluorophore and achieving optical sectioning.

Often, multiple fluorophores are used, with each fluorophore having a different spectra,
some of which may overlap. Typically, the ability to distinguish between the respective
fluorophores is only possible where the excitation and emission spectra are separated, or where
the fluorescence lifetimes are distinct.

20 Another approach is to selectively excite different fluorophores by using various
excitation photon wavelengths, each of which will approximate the wavelength of the absorption
peak of a corresponding fluorophore. Such an approach is not practical for a number of reasons.
Firstly, it is difficult to rapidly tune the excitation wavelength of the laser providing the
excitation photons. Secondly, there is typically a very broad excitation spectrum, so that such
25 an approach makes it difficult to excite a single dye.

Thus, the need exists for a system and method for efficiently monitoring a plurality of
fluorescent probes, and to selectively record the signal from those probes for subsequent analysis.
The present invention addresses these needs.

30 SUMMARY OF THE INVENTION

Briefly, the present invention is directed to a system and method for monitoring cellular
activity in a cellular specimen. According to one illustrative embodiment of the invention, a
plurality of excitable markers are applied to the specimen. A multi-photon laser microscope is
provided to excite a region of the specimen and cause fluorescence to be radiated from the
35 region. The radiating fluorescence is processed by a spectral analyzer to separate the
fluorescence into wavelength bands. The respective fluorescence bands are then collected by an
array of detectors, with each detector receiving a corresponding one of the wavelength bands.

1 According to another embodiment, the invention is directed to a system for monitoring
cellular activity in a cellular specimen that contains a plurality of excitable markers. The system
includes a laser microscope that is operative to excite the markers in a region of the specimen,
5 so that those markers in the region radiate fluorescence. The system also includes a tunable filter
that is operative to process the fluorescence and to pass a portion of the fluorescence wavelengths
radiated by the markers. The system still further includes a detector that is operative to receive
the processed fluorescence wavelengths.

10 In still another embodiment, the invention is directed to a system for monitoring cellular
activity, including a two-photon laser microscope that is operative to excite the markers in a
region of the specimen such that the markers in the region radiate fluorescence. The system also
includes a detector that is operative to receive non-descanned fluorescence from the specimen.

BRIEF DESCRIPTION OF THE DRAWINGS

15 FIG. 1 is a schematic diagram of a first illustrative embodiment of the present invention;
FIG. 2 is a schematic diagram that illustrates one embodiment of a spectral analyzer
included in the system of FIG. 1;

FIG. 3 is a schematic diagram that illustrates a system according to another illustrative
embodiment of the invention;

20 FIG. 4 is a schematic diagram that illustrates yet another illustrative embodiment of the
invention;

FIG. 5 is a schematic diagram that illustrates still another embodiment of the invention;

FIG. 6 is a flow chart that depicts the operational flow of processing software to process
spectral data received by the systems of any of FIGS. 1 through 5 according to one illustrative
embodiment of the invention;

25 FIGS. 7a-c depict emissions spectra for various fluorescent dyes; and

FIG. 7d depicts a measured spectrum from a specimen that contains plural different dyes.

DETAILED DESCRIPTION OF THE INVENTION

30 Referring to FIG. 1, a novel system 10 is disclosed for capturing spectral data from a
specimen 12 to which a plurality of excitable markers have been applied. In one embodiment,
at least two fluorescent dyes (i.e., fluorescent probes) are applied to the specimen. The
fluorescent dyes have respective emission spectra that may or may not overlap. In the case where
the emission spectra are very similar, it becomes difficult to separate out the emission spectra to
35 determine the contributions of each of the fluorescent dyes to the observed spectra. System 10
facilitates making such determinations in an efficient, reliable manner.

As shown in FIG. 1, system 10 includes a laser 14 that generates laser light. The light

1 emitted by laser 14 is focused by a lens 16 onto a short pass dichroic mirror 18. As is well known, the dichroic mirror 18 selectively reflects light according to its wavelength. Thus, the dichroic mirror is selected such that it reflects the light emitted by laser 14.

5 The reflected light from dichroic mirror 18 is directed toward scanning optics, shown schematically at 20. The scanning optics may include a controllable deflection unit, or any other suitable structure that allows the reflected light to be redirected for scanning of the specimen 12 by the laser light, as is well known to those skilled in the art.

10 The directed light from scanning optics 20 is imaged by a microscope lens 22 onto or into the specimen 12. The laser light excites the fluorescent dyes in the region where the light is directed, such that those dyes fluoresce and emit light having respective emissions spectra.

15 In one embodiment, laser 14 is a two-photon laser. As is well known, a two-photon laser microscope depends on the two-photon effect, by which the fluorescent dyes are excited not by a single photon, but rather by two relatively low energy photons that are absorbed contemporaneously by a fluorescent dye. Thus, the requirement for two coincident (or near coincident) photons to achieve excitation of the fluorescent dye means that only focused light reaches the required intensities and that scattered light does not cause excitation of the fluorescent dyes. As such, a two-photon laser microscope is inherently insensitive to the effects of light scattering in thick slices. Alternatively, the laser 14 may comprise some other multi-photon laser, or a single-photon laser.

20 The fluorescence radiated by the excited dyes is focused by the microscope lens 22, and passes through the scanning optics 20 and dichroic mirror 18. The fluorescence is then focused by a lens 24 and directed to a light guide 26 that delivers the focused light to a spectral analyzer 28. Spectral analyzer 28 receives the light and disperses the light into respective wavelength bands. Each band is detected by a corresponding detector, and the intensity of each band is recorded and processed to determine the ingredients of the received fluorescence, as is described in more detail below in connection with FIG. 2.

25 Referring to FIG. 2, there is shown one illustrative embodiment of spectral analyzer 28. Spectral analyzer 28 includes a housing 30 formed with a light entrance opening 32 and a light exit opening 34. Light entrance opening 32 is connected to light guide 26 to receive light from the light guide. Preferably, the distal end of light guide 26 includes an exit slit 27 to permit the delivery of fluorescence from light guide 26 to spectral analyzer 28. Mounted within spectral analyzer 28 is a first mirror 36 that directs the incoming light to a grating 38, which disperses the light over appropriate wavelength range. The dispersed light is then directed to a second mirror 35 40 that directs the dispersed light through the exit opening 34. In place of the grating 38, spectral analyzer 28 may alternatively include a prism or other light-dispersing structure.

1 Aligned with exit opening 34 is a detector array 42 consisting of individual detectors 43.
The detector array 42 may take many different forms, such as an array of photomultiplier tubes
(PMTs), multiple window PMTs, position-dependant wire detectors, position/time-sensitive
5 detectors, a photodiode array, an intensified photodiode array, charge-coupled devices (CCDs),
intensified CCDs, an SIT or other video camera, or any other suitable optical-to-electrical
transducer.

10 In any event, the detector array 42 is preferably a linear array, with the respective
wavelength bands being incident upon a corresponding one of the detectors 43. Each detector
43 in the array receives the light incident upon it and generates a corresponding analog electrical
signal. The electrical signals are then introduced to respective analog-to-digital converters
(shown schematically at 44) which convert the incoming analog signals into corresponding
digital signals. The digital signals are then delivered to a processor 46 that processes the digital
15 signals to determine the constituents of the emissions spectra, as is described in more detail
below.

20 Referring now to FIG. 3, there is shown a system 50 according to an alternative
embodiment of the invention. In place of spectral analyzer 28, system 50 includes a tunable filter
52 that is interposed between lens 24 and a single detector 54, for example, a PMT. In one
embodiment, tunable filter comprises a liquid crystal tunable filter (LCTF) that utilizes liquid
crystals to continuously vary the retardance of individual filter stages, resulting in a narrow band
filter that is electrically tunable over a wide spectral range. Alternatively, the tunable filter 52
may comprise an acousto-optical tunable filter. In any event, tunable filter is controlled by a
suitable control unit 56 to vary the bandpass of the filter through the spectral range. At each
25 bandpass, detector 54 receives fluorescence and generates a corresponding electrical output
signal, which is converted to digital format by an analog-to-digital converter 58 and then
introduced to processor 46 for processing.

30 Referring now to FIG. 4, there is shown a system 60 according to still another
embodiment of the invention. System 60 includes a long pass dichroic mirror 62 placed in the
light stream between the scanning optics 20 and the microscope lens 22. The dichroic mirror 62
is selected such that wavelengths corresponding to the fluorescence radiated by the specimen are
reflected by the mirror 62, while the laser light from laser 14 passes through without being
reflected. The reflected fluorescence is directed to a focusing lens 64, and then introduced to
light guide 26 which delivers the light to spectral analyzer 28. As described above, spectral
35 analyzer disperses the light and passes the light on to ADC 44, which converts the respective
bands into digital signals and introduces the digital signals to processor 46.

System 60 is therefore suitable for use in connection with a non-descanned two-photon

1 microscope. By diverting the radiated fluorescence before it passes through the scanning optics
20, a signal of increased intensity is received by spectral analyzer 28, as compared with a signal
that passes through scanning optics 20 and dichroic mirror 18 before being received by a spectral
5 analyzer or detector. Thus, the dwell time at each pixel can be reduced as a result.

Referring now to FIG. 5, a system 70 is shown according to another embodiment of the
invention. System 70 includes a light collector 72 that substantially surrounds specimen 12 and
includes a reflective inner surface. Thus, light emitted by fluorescent dyes within specimen 12
are collected by the collector, regardless of the direction in which the light radiates. In one
10 embodiment, collector 72 comprises an integrating sphere. Alternatively, collector 72 may be
in the shape of an ellipsoid or other structure that substantially encompasses specimen 12 to
collect a substantial amount of the light radiating from specimen 12, for example, an elliptical
mirror. Collector 72 connects to light guide 26 to deliver the collected light to spectral analyzer
28.

15 Thus, in use of the various systems described above, laser light is directed by dichroic
mirror 18, scanning optics 20, and focusing lens 22 to a region of specimen 12. The photons
(either from a single-photon laser or from a multi-photon laser) excite the fluorescent dyes in the
region, causing them to fluoresce. The entire emitted spectrum is received and processed
20 simultaneously in certain of the illustrative embodiments to speed up the collection process. The
spectral information is then processed to determine the amounts of each dye contributing to the
emitted spectrum.

Referring now to FIG. 6, operation of processor 46 is described in greater detail. In one
embodiment, processor 46 is programmed to execute a linear unmixing operation upon the
25 incoming spectral data to approximate the quantities of each fluorescent dye that contributed to
the emitted spectrum. As shown in FIG. 6, operation begins at step 100 by determining spectral
characteristics for the respective individual fluorescent dyes. As is well known in the art, each
of the fluorescent dyes emits a particular spectrum over a certain wavelength band and at certain
varying intensities within that band (see FIGS. 7a-c which illustrate examples of emissions
30 spectra for three different fluorescent dyes). At step 102, data relating to the probe spectra is
recorded in processor memory for subsequent retrieval.

At step 104, processor 46 receives the measured imaging spectrum data from ADC 44
(see FIG. 7d), and retrieves the characteristic spectra for the various fluorescent dyes. At step
106, the measured spectrum is decomposed into the various component dye spectra. This can
35 be accomplished in many different ways. In one embodiment, processor 46 generates a model
spectrum from the individual templates, and compares the model spectrum with the actual
recorded spectrum. The respective weights of each of the individual templates are then varied

1 to arrive at a close approximation of the actual spectrum. Then, at step 108, processor 46 determines the weights of each dye to quantitate the respective dye intensities.

5 In another embodiment, processor 46 is programmed to carry out principal component analysis (PCA) on the incoming data. As is well known in the art, PCA is a linear model which transforms the original variables of an emission spectrum into a set of linear combinations of the original variables called principal components, that account for the variance in the original data set. Suitable forms of PCA algorithms are disclosed in U.S. Patent Numbers 5,991,653 to Richards-Kortum et al., and 5,887,074 to Lai et al., the disclosures of which are hereby expressly
10 incorporated by reference.

Preferably, system 10 uses a single excitation to excite the respective dyes (i.e., the dyes are pan-excited). In this manner, all of the dyes are excited at once by using a single wavelength. Thus, there is no need for using more than one wavelength, nor is there a need to take multiple images and overlay the respective pixel images to generate a complete image. Alternatively,
15 system 10 may use multiple excitations, such as two excitations, to excite the dyes, especially in situations where it is desirable to cover a relatively broad span of dyes.

From the foregoing, it will be apparent to those skilled in the art that the present invention provides an efficient and reliable system for receiving and processing emissions spectra in connection with fluorescence microscopy. The system processes the emissions spectra to
20 determine concentrations of plural fluorescent dyes in a particular spectrum, even where the wavelength bands of the dyes overlap.

While the above description contains many specific features of the invention, these should not be construed as limitations on the scope of the invention, but rather as one exemplary embodiment thereof. Many other variations are possible. Accordingly, the scope of the
25 invention should be determined not by the embodiments illustrated, but by the appended claims and their legal equivalents.

1 What is claimed is:

1. A method of monitoring cellular activity in a cellular specimen, comprising:
applying a plurality of different excitable markers to the specimen;
applying light to the specimen from a multi-photon laser microscope to excite a
region of the specimen and cause fluorescence to be radiated from the region by the markers in
that region;
separating the fluorescence into wavelength bands using a spectral analyzer; and
detecting the fluorescence through an array of detectors, with each detector
receiving one of the wavelength bands and generating a corresponding signal.
2. The method of claim 1, wherein separating the fluorescence includes using a
grating.
3. The method of claim 1, wherein separating the fluorescence includes using a
prism.
4. The method of claim 1, wherein separating the fluorescence includes using a
liquid crystal tunable filter.
5. The method of claim 1, wherein separating the fluorescence includes using an
acousto-optical tunable filter.
6. The method of claim 1, wherein applying a plurality of excitable markers includes
applying a plurality of fluorescent probes to the specimen.
7. The method of claim 1, wherein detecting the fluorescence includes using a
plurality of photomultiplier tubes.
8. The method of claim 1, wherein detecting the fluorescence includes using a
plurality of high gain photomultiplier tubes.
9. The method of claim 1, wherein applying light to the specimen comprises
applying light from a two-photon laser microscope.
10. A system for monitoring cellular activity in a cellular specimen that contains a
plurality of excitable markers, the system comprising:
a laser microscope that is operative to excite the markers in a region of the
specimen, wherein the markers in the region radiate fluorescence as a result;
a tunable filter that is operative to process the fluorescence and to pass a portion
of the fluorescence, wherein the portion of the fluorescence is within a wavelength band that

1 depends on the setting of the filter; and

a detector that is operative to receive the processed fluorescence and to convert the fluorescence into a corresponding signal.

5 11. The system of claim 10, wherein the tunable filter comprises a liquid crystal tunable filter.

12. The system of claim 10, wherein the tunable filter comprises an acousto-optical tunable filter.

10 13. The system of claim 10, wherein the detector comprises a photomultiplier tube.

14. The system of claim 10, wherein the detector comprises a high-gain photomultiplier tube.

15 15. The system of claim 10, wherein the laser microscope comprises a multi-photon laser microscope.

16. A system for monitoring cellular activity in a cellular specimen that contains a plurality of excitable markers, the system comprising:

20 a multi-photon laser microscope that is operative to excite the markers in a region of the specimen, wherein the markers in the region radiate fluorescence as a result;

a deflector positioned relative to the specimen to deflect non-descanned fluorescence radiated by the markers;

a spectral analyzer operative to receive the deflected, non-descanned fluorescence from the deflector and to disperse the fluorescence; and

25 a detector array that is operative to receive the dispersed fluorescence from the spectral analyzer and to generate corresponding signals.

17. The system of claim 16, wherein the detector array comprises a plurality of photomultiplier tubes.

30 18. The system of claim 16, wherein the detector array comprises a plurality of high-gain photomultiplier tubes.

19. A method of monitoring cellular activity in a cellular specimen, comprising:

applying a plurality of different excitable markers to the specimen;

35 focusing light upon a region of the specimen from a laser microscope to excite the markers in the region and cause fluorescence to be radiated by the markers in the region;

separating the fluorescence into wavelength bands;

1 detecting the fluorescence through an array of detectors, with each detector receiving one of the wavelength bands and generating a corresponding signal; and

5 processing the signals from the detectors to calculate the quantities of each marker in the region.

20. The method of claim 19, wherein separating the fluorescence includes using a grating.

10 21. The method of claim 19, wherein separating the fluorescence includes using a prism.

22. The method of claim 19, wherein separating the fluorescence includes using a liquid crystal tunable filter.

15 23. The method of claim 19, wherein separating the fluorescence includes using an acousto-optical tunable filter.

24. The method of claim 19, wherein applying a plurality of excitable markers includes applying a plurality of fluorescent probes to the specimen.

25. The method of claim 19, wherein collecting the fluorescence includes using a plurality of photomultiplier tubes.

20 26. The method of claim 19, wherein collecting the fluorescence includes using a plurality of high gain photomultiplier tubes.

27. The method of claim 19, wherein applying light to the specimen comprises applying light from a two-photon laser microscope.

25 28. The method of claim 19, wherein processing the signals comprises performing linear unmixing on the signals.

29. A system for monitoring cellular activity in a cellular specimen that contains a plurality of excitable markers, the system comprising:

30 a laser microscope that is operative to excite the markers in a region of the specimen, wherein the markers in the region radiate fluorescence as a result;

a collector that at least substantially envelops the specimen to receive fluorescence from the markers;

35 a spectral analyzer connected to the collector to receive the fluorescence and process same to disperse the fluorescence; and

a detector that is operative to receive the dispersed fluorescence and to convert the fluorescence into corresponding signals.

1

30. The system of claim 29, wherein the collector comprises an integrating sphere.

31. The system of claim 29, wherein the detector comprises an array of
photomultiplier tubes.

5

32. The system of claim 29, wherein the detector comprises an array of high-gain
photomultiplier tubes.

33. The system of claim 29, wherein the laser microscope comprises a multi-photon
laser microscope.

10

15

20

25

30

35

1/7

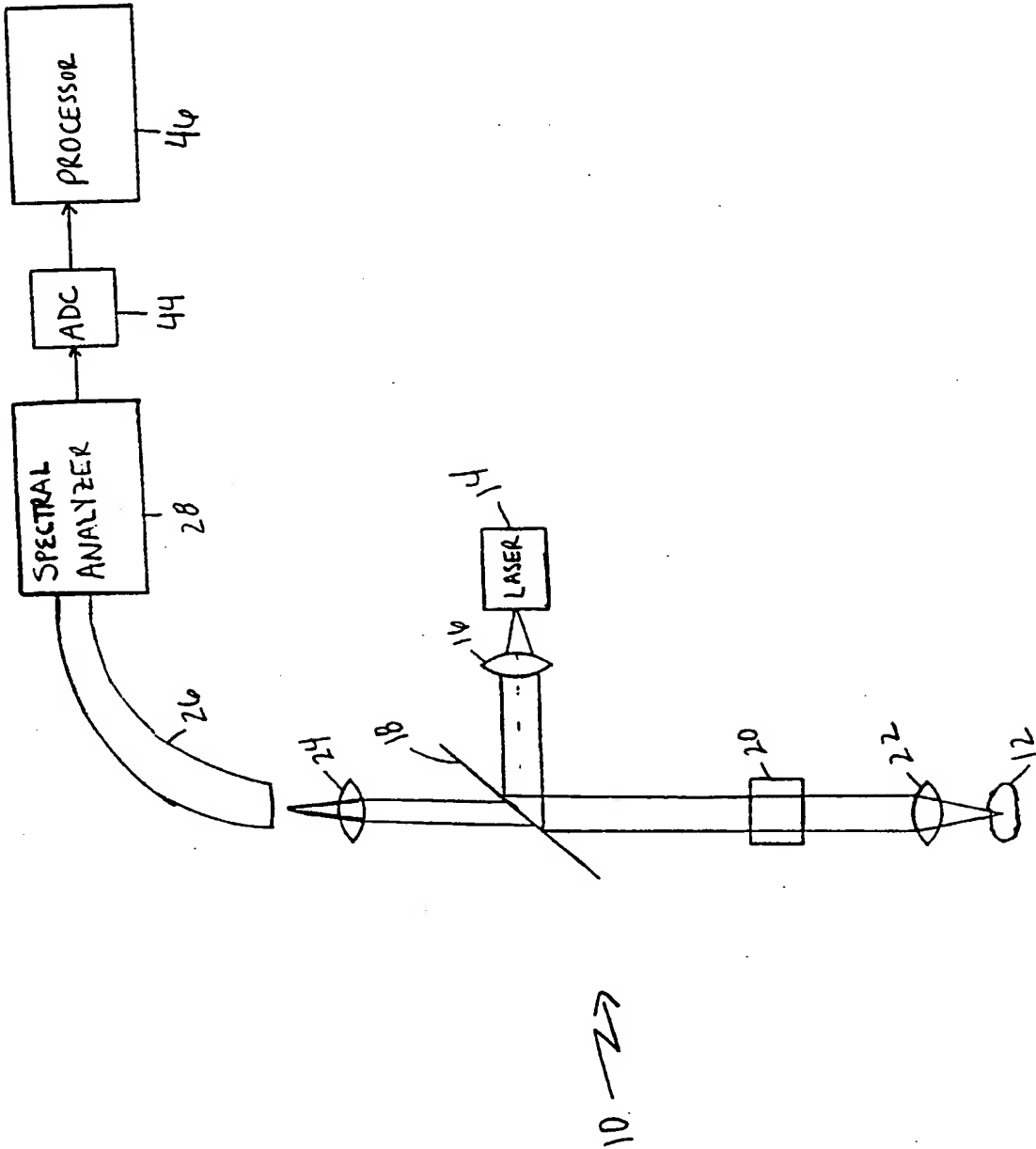


FIG. 1

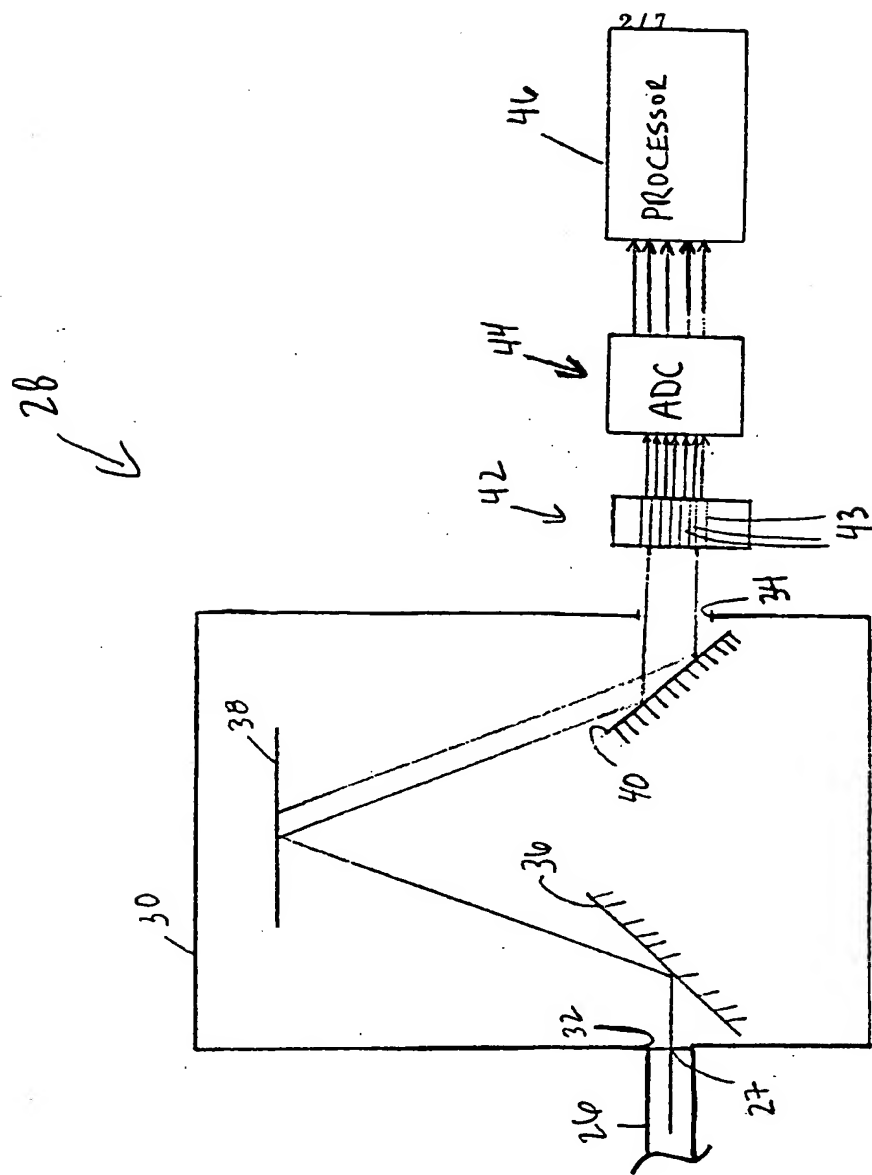
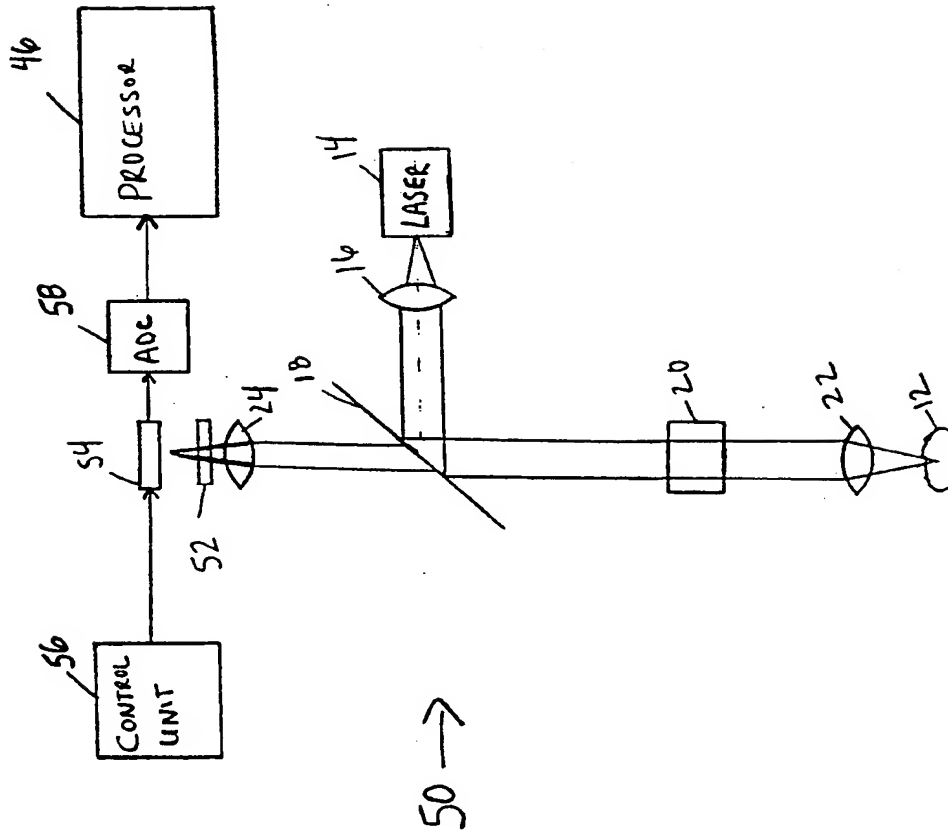


Fig. 2

FIG. 3



4/7

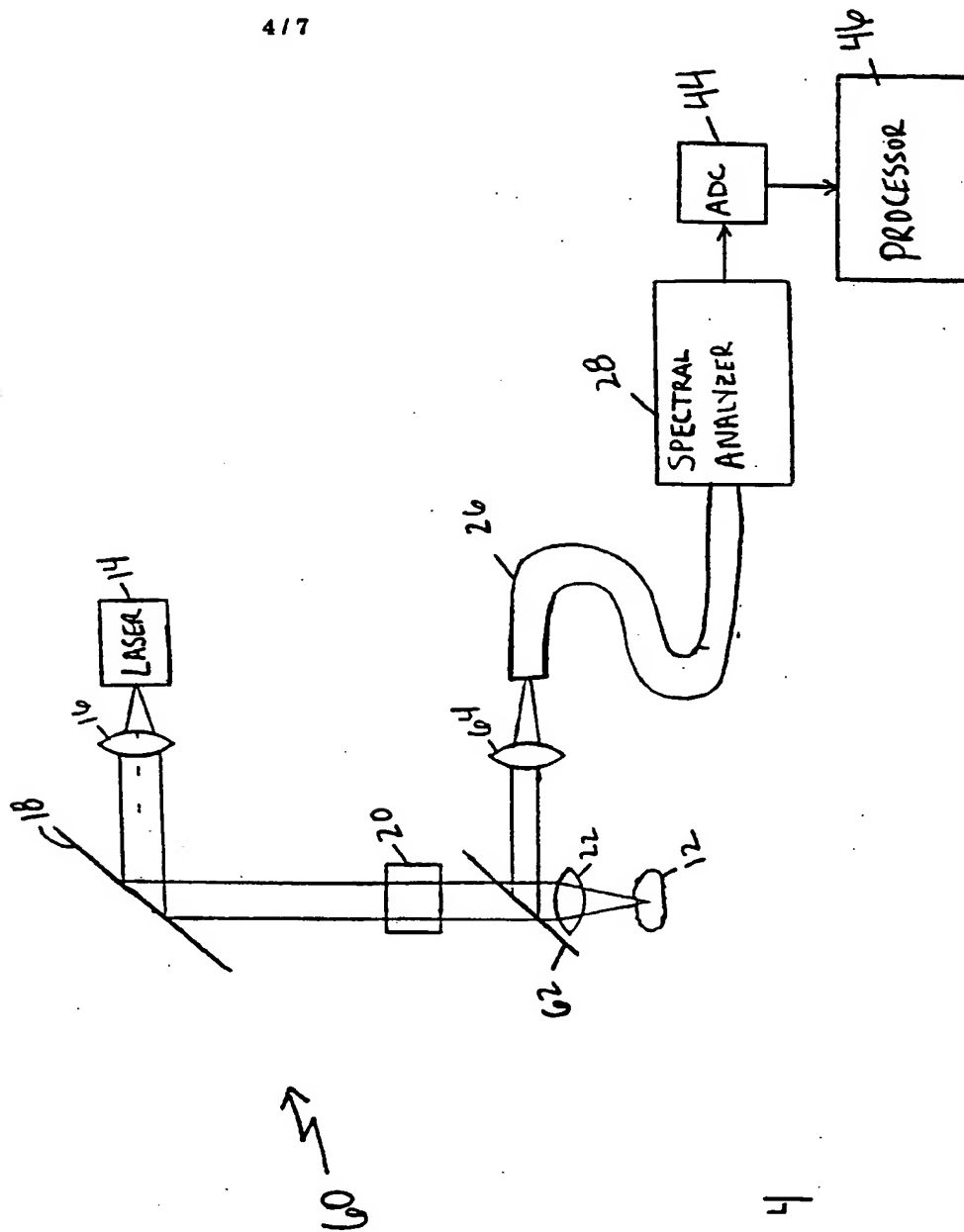
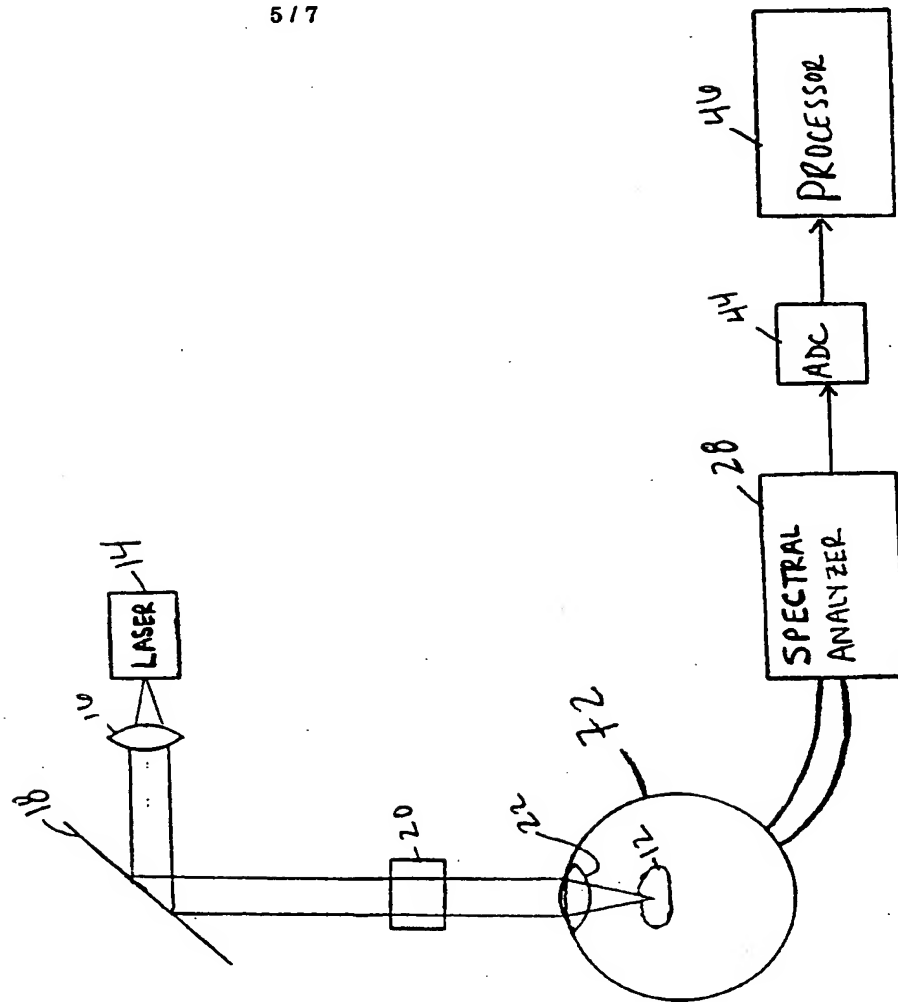


FIG. 4

5/7



70 →

FIG. 5

6/7

FIG. 6

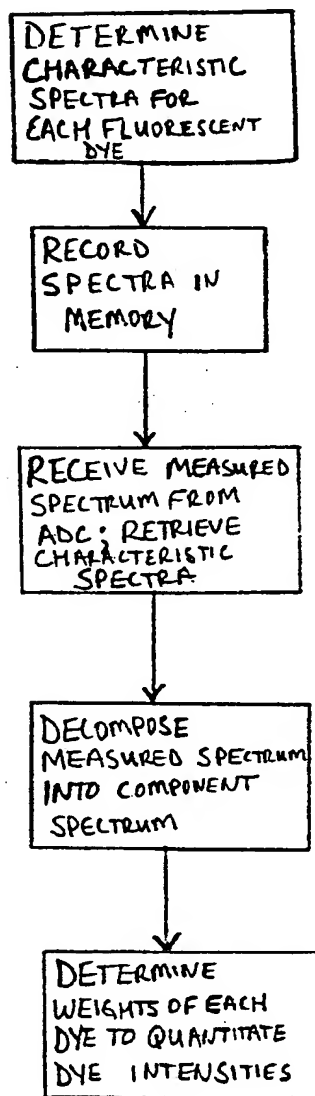


FIG. 7a 7/7

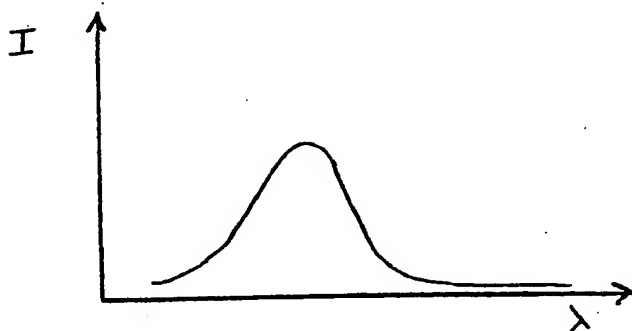


FIG. 7b

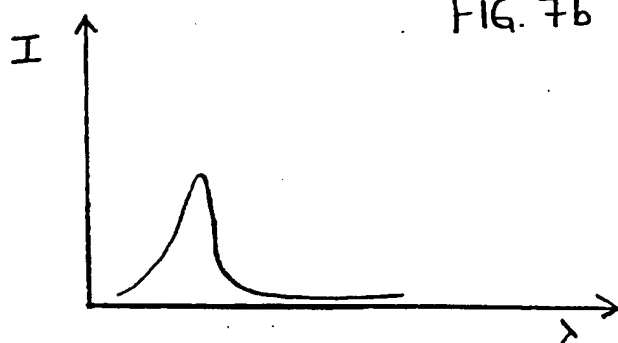


FIG. 7c

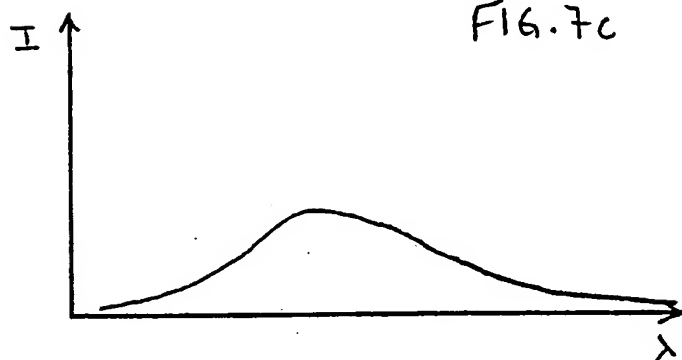
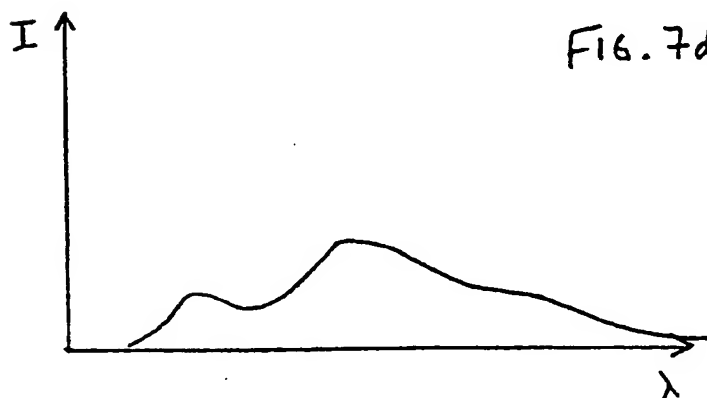


FIG. 7d



A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N21/64 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, INSPEC, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| X | WO 96 22521 A (SOINI ERKKI ;HAENNINEN PEKKA (FI)) 25 July 1996 (1996-07-25) page 14; claims 1-3; figure 1 | 1-33 |
| A | US 5 891 738 A (SOINI ERKKI ET AL) 6 April 1999 (1999-04-06) column 15, line 40 - line 55 | 1-33 |
| A | US 5 674 743 A (ULMER KEVIN M) 7 October 1997 (1997-10-07) figure 9 | 1-33 |
| A | EP 0 916 981 A (MAX PLANCK GESELLSCHAFT) 19 May 1999 (1999-05-19) figure 3 | 1-33 |
| | --- -/-- | |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

28 November 2000

Date of mailing of the international search report

06/12/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mason, W

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | US 4 905 169 A (BUICAN TUDOR N ET AL) 27 February 1990 (1990-02-27) figure 7 | 1-33 |
| A | US 5 117 466 A (BUICAN TUDOR N ET AL) 26 May 1992 (1992-05-26) claim 1 | 1-33 |
| A | US 5 814 820 A (DONG CHEN-YUAN ET AL) 29 September 1998 (1998-09-29) figure 5 | 1-33 |
| A | WO 99 37999 A (WISCONSIN ALUMNI RES FOUND) 29 July 1999 (1999-07-29) figure 1 | 1-33 |

| Patent document cited in search report | | Publication date | Patent family member(s) | Publication date |
|---|---|---------------------|---|--|
| WO 9622521 | A | 25-07-1996 | FI 950174 A EP 0804723 A JP 10512952 T | 17-07-1996 05-11-1997 08-12-1998 |
| US 5891738 | A | 06-04-1999 | FI 96641 B FI 951040 A EP 0804732 A WO 9622531 A JP 10512670 T EP 0815447 A WO 9627798 A JP 11503824 T | 15-04-1996 08-09-1996 05-11-1997 25-07-1996 02-12-1998 07-01-1998 12-09-1996 30-03-1999 |
| US 5674743 | A | 07-10-1997 | AU 1228197 A AU 673245 B AU 6131694 A CA 2155186 A EP 0682671 A JP 8506664 T MX 9400837 A WO 9418218 A | 27-03-1997 31-10-1996 29-08-1994 18-08-1994 22-11-1995 16-07-1996 31-08-1994 18-08-1994 |
| EP 0916981 | A | 19-05-1999 | JP 11249023 A US 6128077 A | 17-09-1999 03-10-2000 |
| US 4905169 | A | 27-02-1990 | NONE | |
| US 5117466 | A | 26-05-1992 | NONE | |
| US 5814820 | A | 29-09-1998 | NONE | |
| WO 9937999 | A | 29-07-1999 | AU 2339899 A GB 2349033 A | 09-08-1999 18-10-2000 |